BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

(12) UK Patent Application (19) GB (11) 2 209 468 (13) A

(43) Date of A publication 17.05.1989

(21) Application No 8821105.7

(22) Date of filing 08.09.1988

(30) Priority data
(31) 8721108 (32) 08.09.1987 (33) GB

(71) Applicant
The University of Salford
Salford, Manchester, M5 4WT, United Kingdom

(72) Inventor
Christopher Grant Morgan

(74) Agent and/or Address for Service
Marks & Clerk
Suite 301, Sunlight House, Quay Street, Manchester, M3 3JY, United Kingdom

(51) INT CL⁴
A61K 9/50, B01J 13/02

(52) UK CL (Edition J)
A5B BLH B829

(56) Documents cited
EP 0199362 A
Methods Enzymol. 149 (Drug Enzyme Targeting, Pt. B), 99-111 (1987)
Proc. Natl. Acad. Sci. U.S.A. 84(1), 246-50 (1987)
Br. J. Cancer 48(2), 307-9 1983
Photochem. Photobiol 37(5), 491-4 (1983)
Photochem. Photobiol 24(1), 41-8 (1976)

(58) Field of search
UK CL (Edition J) A5B BLH, B8C CA
INT CL⁴ A61K, B01J

(54) Photosensitive liposomes

(57) Liposomes with an incorporated photosensitising agent or agents and which are such that absorption of light of the appropriate wavelength results in destabilisation of the lipid bilayer and fusion between liposomes and/or exchange of membrane bound constituents of the liposomes between liposomes and/or cells or tissues of a recipient of the liposomes and/or fusion of intact liposomes with such cells or tissues.

The liposomes are particularly useful as vehicles for pharmaceutical or cosmetic compounds.

GB 2 209 468 A

2209463

LIPOSOMES

The present invention relates to liposomes, and more particularly although not exclusively to pharmaceutical and/or cosmetic compositions comprising such liposomes.

For many drugs significant side effects must be tolerated if a clinically effective dose is given. Problems of this type are especially marked with many agents used in cancer chemotherapy, to the extent that treatment is sometimes discontinued. One of the most promising approaches to the minimisation of side effects is the localised delivery of a drug, so that high drug concentrations are achieved only where needed. Drug delivery systems, designed to 'target' drugs to specific sites, are under intensive investigation and development at the present time.

One 'vehicle' for drug delivery is the liposome. Liposomes are formed spontaneously when phospholipids are dispersed in aqueous media, and comprise an inner aqueous space enclosed by a phospholipid bilayer. Liposomes can be unilamellar, or can be multilamellar with many concentric bilayers and aqueous compartments, depending on methods of preparation. Water-soluble drugs can be entrapped within the aqueous interior of liposomes, and lipid-soluble drugs can be incorporated within the bilayer itself. The stability of liposomes in vivo and the fate of trapped contents depends on liposome composition, size and charge, and a very large literature exists describing the effects of these variables. For detailed reviews of all aspects of liposomes, including those relevant to drug delivery, see Liposome Technology Vol. III CRC Press 1984 G. Gregoriadis ed.

Liposomes injected intravenously are taken up by macrophages of the reticuloendothelial system (RES), and liposomes localise predominantly in liver, spleen and bone marrow. In this respect, liposomes are 'passively' targetted to these organs, and this targetting has clinical application. Many workers have tried to achieve 'active' targetting to other tissues, but to date these attempts have met with little success except for binding of liposomes to cells in vitro. A common strategy is to conjugate 'recognition' molecules to the liposome, for example antibodies directed against tumour cell membranes. There are several reasons why efforts in vivo so far have proved disappointing. One problem is rapid uptake by the RES, limiting circulatory half lives for liposomes, and minimising the probability of liposomes finding target sites. This uptake depends on liposome charge, size and composition, and these can be tailored to alter patterns of distribution. In addition, the reticuloendothelial system can be temporarily 'blockaded' by injection of large quantities of inert liposomes before administration of drug-containing liposomes. This aspect therefore offers no insurmountable problem in principle.

A second problem with targetting strategies in vivo is the lack of well defined tumour-specific antigens, and the ability of many tumours to shed antigens into the circulation. This aspect can be approached by studying patterns of antigenic distribution, and preparing liposomes with several antibodies on the surface.

Another major problem is accessibility of liposomes to extravascular target cells. There is little evidence that liposomes can cross capillaries, except for the discontinuous capillaries of the liver

and other RES sites. This is one of the primary reasons for 'passive' targetting to these sites.

Drugs may also be applied topically, and liposomes are currently under investigation as topical delivery agents for pharmaceuticals and cosmetic agents such as anti-oxidants.

An alternative approach to targetted drug delivery is incubation of liposomes, with a sample of cells from a patient which are subsequently returned (to the patient) after treatment. For example, antibody targetted liposomes can bind to and kill tumor cells in a bone marrow or blood sample.

Even where liposomes can be targetted successfully to a given cell type (e.g. by injection directly into target sites, topical application or by incubation with cells in -vitro), liposomes are not always taken up into the cells, and liposomal contents are not necessarily released. Considerable ingenuity has been invested in attempts to 'trigger' release from liposomes at target sites. To date, this has largely concentrated on release of soluble entrapped materials. One approach has used liposomes which leak contents on heating through a critical temperature range, in conjunction with radiofrequency heating of target tissues (Design of liposomes for enhanced local release of drugs by hyperthermia. Yatvin, M.B., Weinstein, J.N., Denniss, W.H. and Blumenthal, R. Science 202 1290 (1978)). Another approach has used liposomes prepared from pH-sensitive lipids, which leak contents in regions of low pH, such as are often associated with tumours (pH-sensitive liposomes..possible clinical implications Yatvin, M.B., Kreutz, W., Horwitz, B.A. and Shinitzky, M. Science 210 1253 (1980)). Both of these approaches have aroused very considerable

interest and are widely quoted. It is clear that externally triggered drug delivery to target sites is a very desirable objective.

There are many tissues which can be illuminated with high intensity light using lasers and fibre-optic technology. Phototherapy of tumours has already been successfully achieved using photosensitising drugs related to bile pigments (The future of photoradiation therapy in the treatment of cancer T. J. Dougherty Laser Focus 55-58 (July 1983)). Patients are given doses of a mixture of haematoporphyrin derivatives, which appear to be effectively taken up by tumours. Illumination causes cell damage by a mechanism thought to be primarily mediated by toxic oxygen radicals (Spikes J. D. in The Science of Photobiology 87-112 Plenum (1977)). Patients having taken the drug must stay out of sunlight for about one month, but otherwise side effects are rare. This appears to be a very promising approach to the treatment of localised cancers in areas where surgery is difficult. The porphyrin derivatives used are primarily sensitive to short wavelength light, but have sufficient absorbance at longer wavelengths for red light to be used for activation. It is generally desirable to use long wavelength light where possible, since this has a greater depth of penetration into tissues than do the short wavelengths.

Liposomal delivery of porphyrins has been investigated for phototherapy mediated by toxic oxygen species and other free radicals.

Additionally light induced permeability changes of sensitised liposomal membranes have been reported to allow loss of entrapped water soluble species, and application to delivery of water soluble drugs

to illuminated regions has been suggested (Pidgeon, Charles and Hunt, C. Anthony "Light Sensitive Liposomes" Photochemistry and Photobiology 37(5) 1983 491-494)

According to a first aspect of the present invention there is provided liposomes with an incorporated photosensitising agent or agents and which are such that absorption of light of the appropriate wavelength results in destabilisation of the lipid bilayer and fusion between liposomes and/or exchange of membrane constituents of the liposomes between liposomes and/or cells or tissues of a recipient of the liposomes and/or fusion of intact liposomes with such cells or tissues.

The invention also provides a method in which liposomes as defined in the preceeding paragraph are irradiated with light of the appropriate wavelength so as to effect said destabilisation of the lipid bilayer.

The liposomes of the invention are particularly useful as delivery vehicles for pharmaceuticals or cosmetics and accordingly the invention also provides a pharmaceutical or cosmetic composition comprising liposomes as defined above incorporating a pharmaceutical or a cosmetic agent (or a precursor of either such agent), preferably within the bi-layer.

Liposomes which are capable, upon irradiation, of fusing together and/or with cells or tissues, and/or exchange of membrane bound constituents either with other liposomes or with cells or tissues have a number of important advantages. These may be summarised briefly as follows:

Liposomes which fuse together upon irradiation have the advantage that liposomal size changes, which may alter clearance rates from tissues.

Additionally, agents separately bound to sub-sets of a mixture of liposomes become mixed on liposome fusion. Mixing of precursors might be used, for example, to generate active materials from 'pro-drug' precursors which separately are of lower activity or might allow co-storage of materials which are incompatible or which react together to yield products of short lifetime or otherwise limited stability.

Liposomes which fuse to cells or tissues have the advantage that agents could be transferred directly from liposomes into cells for therapeutic or other purposes such as to alter gene expression within such cells, for example, materials such as DNA or enzymes might be transferred into cells by this means.

Liposomes which transfer membrane bound constituents have the advantage that lipophilic agents might be transferred into cells by contact with such liposomes under controlled conditions, or lipophilic drug or cosmetic derivatives might exchange through solution into cellular membranes when and where such a process is photostimulated.

The destabilisation of the liposomal structure may also alter the permeability of the liposomal membrane to solutes, allowing release of entrapped materials from the aqueous space or spaces within.

Various possibilities exist for providing the change in bilayer stability, which is preferably effected other than by free-radical or oxidative damage thereto. The preferred method is to use a bilayer sensitised with a compound having two

photoisomerisable groups, (the "photoisomerable compound") which, prior to irradiation, provide a stable bilayer structure but which upon irradiation isomerise to a form which destabilises bilayer packing. Preferably the photoisomerable compound comprises two neighbouring chains each with a unsaturated group which, prior to irradiation, is in the trans form and which is converted to the cis form upon irradiation so that there is steric hindrance between the chains resulting in the destabilisation of bilayer packing. Preferably the unsaturated groups are azo groups each conjugated to two aromatic nuclei. Most preferably the photoisomerable compound is a phospholipid incorporating chains with azo groups as aforesaid.

It is preferred that the bilayer is of a gel phase lipid, e.g. a dipalmitoylphosphatidyl-choline.

Liposome compositions of the invention may be used for localised drug delivery by administering the composition to a patient and then irradiating the liposomes at the target site with light of appropriate wavelength to effect the change in bilayer stability. As a result of this stability change there may result fusion of the liposomal membrane with cellular material, or fusion between liposomes affecting a change of rate of clearance of such fused liposomes from the target site, or exchange of lipophilic species between liposomes and tissues or cells.

The liposome may incorporate a drug in the bilayer for release at the target site. A further proposal, however, is that the liposome may contain materials of low toxicity which are converted to more toxic materials only on illumination. Such liposomes would be tolerated at high doses, and repeated administration would be possible.

Liposomal fusion triggered by light absorption offers a means of delivery for drugs or agents which either act synergistically, or which undergo chemical reaction leading to an agent of greater activity than the sum of that of the components. For example, photosensitive liposomes could be used to deliver self-assembling cytotoxins such as those described in the literature (Rideout, D. Science 233 (1986) 561-563).

Lipid solubility is often conferred on otherwise water soluble materials (e.g. drugs) by linkage to an alkyl chain or similar hydrophobic anchor, such that the material can be bound to liposomal carriers through interaction with the liposomal membrane. If the hydrophobic anchor of such a bound material is made photosensitive so that its affinity for the liposomal membrane is reduced on absorption of light of appropriate wavelength, then light-induced detachment of such a material from the liposome becomes possible. This would allow the material to equilibrate between carrier and surrounding cells or tissues. Such a scheme would allow drug delivery for example even in areas where liposomes cannot penetrate the walls of blood vessels or directly interact with target tissues because of size, change or composition.

The inability of intact liposomes to cross capillary boundaries is of little consequence providing light induced destabilisation allows drug exchange to surrounding tissues and sufficiently rapid uptake of released drug is achieved.

Photosensitive liposomes therefore provide a useful adjunct to phototherapy, allowing drugs to interact with locally illuminated sites. One interesting possibility is the use of liposomes to

treat accessible lesions in lung disease. Liposomes can be ingested as aerosols, and are rapidly absorbed into lung tissue, and taken up into macrophages. Alternatively, liposomes injected intravenously are retained effectively in lung capillaries for hours.

The composition of the invention may alternatively be formulated as a delivery vehicle for cosmetic purposes. For cosmetic purposes, the liposomes will be applied topically to enhance skin penetration of agents contained therein on illumination e.g. with near ultra-violet light. Such agents may be anti-oxidants, hormones, moisturisers etc. Similarly topical application of liposomes containing lipophilic pharmaceutical agents might be employed in treatment of skin diseases such as psoriasis or skin cancers.

There are many methods which might be used to photosensitise liposomes. These are summarised in Table 1. For convenience, these have been split up into those which act directly on the liposomal membrane, those where the action on the membrane is mediated by material initially present in the aqueous space, and those which act indirectly <u>via</u> physical effects leading to membrane rupture. The diversity of possible mechanisms for photo-induced release is very important. It allows systems to be designed to optimally match available light sources. In practical applications, this would be a significant factor.

Direct alteration of membrane permeability has been achieved, using liposomes of dipalmitoylphosphatidyl-choline sensitised with a newly synthesised phospholipid which we have called 'Bis-Azo PC'. The structure of Bis-Azo PC and its synthesis is shown in Scheme 1. The azo acid shown

TABLE 1

PHOTOSTIMULATED DRUG DELIVERY

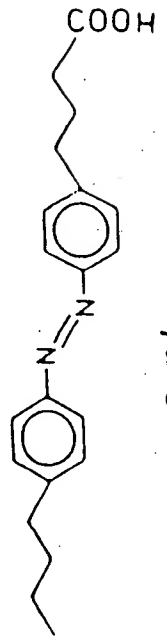
DIRECT	MEDIATED	INDIRECT
Photoisomerisation	Photo-induced ion release (reversible chelation of Calcium or zinc etc.)+	Direct gas release (Diazo etc.)
Photodimerisation of membrane lipid		Dye-sensitised reaction leading to gas release
Photodissociation of head group or acyl chain	Photo-oxidation of membrane (dye sensitised)	Osmotic Pressure Changes
Photoreactivation of blocked lytic toxin or detergent	Dye-sensitised & Depolymerisation (polymeric microspheres)	Photodecomposition of complex Photoinitiated Redox reaction Photocatalysis in homogeneous* phase (enzyme etc.) or heterogeneous phase$

* Homogeneous catalysis might be photoregulated by modulating activity of an effector (e.g. photorelease of ATP from so-called 'caged' ATP), a metal ion (e.g. calcium ion via photoreversible chelators) or other activator/inhibitor...a wide scope exists.

$ Photocatalysis on heterogeneous media has been widely studied in the context of solar energy conversion. Colloidal catalysts have been entrapped within liposomes and have sensitised a variety of reactions including decomposition of 'sacrificial' oxidants and reductants.

+ Liposomes made from charged lipids can be induced to undergo phase transitions leading to fusion and loss of integrity in response to multivalent metal ions. Photoreversible chelators have been synthesised and can be incorporated into liposomes.

& Polymer microspheres offer an alternative to liposomes for drug delivery.



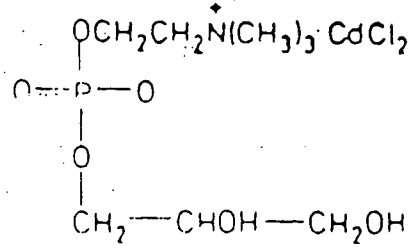
Azo-acid

pivaloyl chloride

tri thylamine

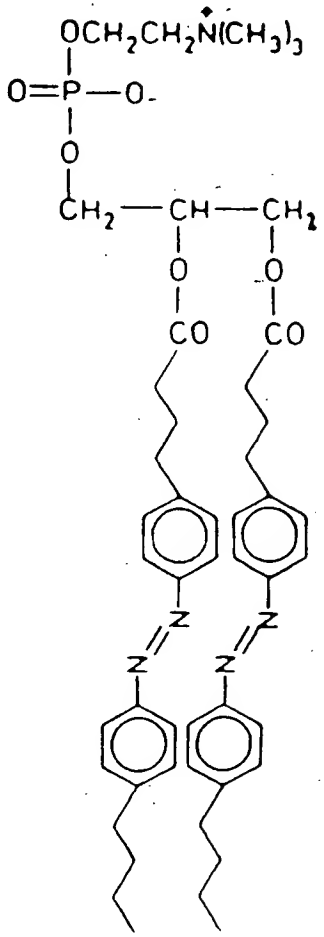
$CHCl_3$

Mixed Anhydride



4-(dimethyamino)pyridine

$CHCl_3$



Bis Azo-PC

Scheme 1

is prepared by reaction of n- butyl nitrosobenzene (from n- butyl aniline by m- chloroperoxybenzoic acid oxidation at 0°C) with 4- aminophenylbutyric acid (from 4- nitro-phenylbutyric acid by catalytic hydrogenation).

The Bis-Azo PC has photoisomerisable azobenzene groups within the acyl chain region. In the all-trans form, the molecule is compatible with close packing of host lipid within a bilayer. On illumination with long-wavelength UV light (UVA), the molecule isomerises to the 'bent' cis form which destabilises bilayer packing thus producing a change of bilayer stability. For this to be effective, we believe it is preferable that both acyl chains of the lipid must be photosensitive: an analogue of Bis-Azo PC having only one photosensitive chain caused no gross perturbation of a bilayer membrane (C. G. Morgan et al. Biochem. Biophys. Acta 820 107-114 (1985)). Liposomes containing Bis-Azo are quite stable at room temperature, and samples containing trapped markers have been stored for weeks in the dark without significant leakage of contents. On illumination with 366nm UV light, the liposomes rapidly fuse together to give larger structures.

Membrane fusion is a very important process, involved in cell-viral interactions and other key biological processes. The ability to trigger fusion photochemically offers a tool to study the time course of primary processes in such fusion, in addition to its practical utility.

As an example of photoinduced liposome fusion, liposomes may be prepared containing Bis-Azo PC in a host bilayer of saturated phospholipid. Liposomes are prepared by co-dissolving a mixture of Bis-Azo PC and dipalmitoyl-L-α-phosphatidylchlorine (DPPC) (in

a typical molar ratio of 1:15 Bis-Azo PC : DPPC) in chloroform and evaporating to give a thin film of mixed lipid. Upon addition of aqueous material and sonication using an ultrasonic probe, small unilamellar liposomes are formed. On illumination with UV light around 366nm from a mercury lamp, the liposomes fuse together. The liposome interaction is first evident as an increase in solution turbidity, followed by eventual precipitation. Electron microscopy shows that liposome size has increased after such illumination, and multilamellar structures are seen.

Further evidence of liposomal interaction is given by experiments where fluorescent molecules are bound to a subset of liposomes. Using chlorophyll -a as fluorescent probe in a liposome sample it is possible to detect fusion of a subset of labelled liposomes with a similar but unlabelled sample by fluorescence changes consequent on photoinduced equilibration of chlorophyll between labelled and unlabelled samples.

For example, inclusion of 1-2 mol% of chlorophyll-a in liposomes prepared as previously described (where the chlorophyll is codissolved in the lipid mixture before solvent evaporation) results in vesicles having low fluorescence in the red, due to intermolecular interactions between chlorophyll molecules. Mixture of such a sample with a tenfold excess of similar liposomes prepared without chlorophyll causes no change in chlorophyll fluorescence, since the molecule cannot equilibrate through solution. However subsequent to ultra violet illumination, enhancement of chlorophyll fluorescence is evident. This is due to dilution of the chlorophyll consequent on lipid mixing.

Control experiments using chlorophyll-a in liposomes prepared without Bis-Azo PC show that this enhancement is a consequence of Bis-Azo PC enhanced lipid mixing, and not due to photodecomposition products of chlorophyll or free radical medicated damage to lipid bilayer integrity.

It is possible to include other components within the lipid bilayer without preventing light induced fusion. Thus, liposomes contained α - tocopherol (DPPC: - tocophenol:Bis-Azo PC molar ratios 85:10:5) similarly fuse on illumination. Cholesterol can also be included at modest concentrations ($\leq$ 10 mole%) - increase of cholesterol decreases the rate of liposome fusion. Increasing amounts of Bis-Azo PC can be incorporated into liposomes, but above about 10 mole % Bis-Azo PC in DPPC host, liposomes are not well sealed to small solutes, which are readily trapped in liposomes containing less sensitiser. On UV irradiation liposomes containing high concentrations of Bis-Azo PC exchange Bis-Azo PC with other liposomes not containing the sensitiser. This is clearly seen from concentration dependent changes in the Bis-Azo PC absorption spectrum, which shifts to shorter wave lengths when high concentrations ($>$ 10% mole) of Bis-Azo PC are present within a subset of liposomes. UV irradiation of such liposomes alone, followed by exposure to blue light establishes a photostationary state which has a characteristic absorption maxiumum. If the experiment is performed with a mixture of such liposomes and unlabelled DPPC liposomes, a shift in absorbance can clearly be seen indicating dilution of Bis-Azo PC. Control experiments show that this process is a transfer of Bis-Azo PC rather than liposome fusion between

Bis-Azo PC containing liposomes and DPPC liposomes. The latter process can occur subsequent to UV irradiation under conditions where transfer to Bis-Azo PC achieves sufficient concentration of the latter within the DPPC liposomes to destabilise these.

CLAIMS

1. Liposomes with an incorporated photosensitising agent or agents and which are such that absorption of light of the appropriate wavelength results in destabilisation of the lipid bilayer and fusion between liposomes and/or exchange of membrane bound constituents of the liposomes between liposomes and/or cells or tissues of a recipient of the liposomes and/or fusion of intact liposomes with such cells or tissues.

2. Liposomes as claimed in claim 1 wherein the bilayer is of a gel phase lipid.

3. Liposomes as claimed in claim 1 wherein the gel phase lipid comprises dipalmitoylphosphatidyl-choline.

4. Liposomes as claimed in any one of claims 1 to 3 wherein the bilayer is sensitised with a compound which upon irradiation undergoes a change of configuration so as to disrupt bilayer stability.

5. Liposomes as claimed in claim 4 wherein said compound incorporates two neighbouring chains each having an unsaturated group which prior to said irradiation is in the trans form and which is converted to the cis form upon said irradiation.

6. Liposomes as claimed in claim 5 wherein said unsaturated group is an azo group.

7. Liposomes as claimed in claim 6 wherein the compound is a phospholipid.

8. Liposomes as claimed in claim 7 wherein the compound is that referred to hereinbefore as Bis-azo PC.

9. A pharmaceutical composition comprising liposomes as claimed in any one of claims 1 to 8 having incorporated therein a pharmaceutical compound

or a precursor thereof together with a pharmaceutically acceptable excipient.

10. A pharmaceutical composition as claimed in claim 9 wherein the pharmaceutical compound or precursor is in the bilayer.

11. A pharmaceutical composition as claimed in claim 10 wherein said compound or precursor is water soluble and is associated with a hydrophobic agent so as to confer lipid solubility to said compound or precursor.

12. A cosmetic composition comprising liposomes as claimed in any one of claims 1 to 8 having incorporated therein a cosmetic agent or precursor thereof together with a cosmetically acceptable excipient.

13. A cosmetic composition as claimed in claim 12 wherein the cosmetic agent is an anti-oxidant, a hormone or a moisturiser.

14. A cosmetic composition as claimed in claim 12 or 13 wherein the cosmetic agent or precursor thereof is in the bilayer.

15. A cosmetic composition as claimed in claim 14 wherein the cosmetic agent or precursor thereof is water soluble and is associated with a hydrophopic agent to confer lipid solubility to said agent or precursor.

16. A method of using liposomes as claimed in any one of claims 1 to 8, a pharmaceutical composition as claimed in any one of claims 9 to 11 or a cosmetic composition as claimed in any one of claims 12 to 15 comprising irradiating the liposomes at said appropriate wavelength.

17. A method as claimed in claim 16 wherein said appropriate wavelength is in the UVA region of the spectrum.

18. Liposomes as claimed in claim 1 substantially as hereinbefore described.

19. A pharmaceutical compositon as claimed in claim 9 substantially as hereinbefore described.

20. A cosmetic composition as claimed in claim 12 substantially as hereinbefore described.

21. A method as claimed in claim 16 of using liposomes substantially as hereinbefore described.

Published 1988 at The Patent Office. State House. 66 71 High Holborn. London. WC1R 4TP. Further copies may be obtained from The Patent Office.